CHAPTER 9. ISOLATION & IDENTIFICATION OF PATHOGENIC *YERSINIA ENTEROCOLITICA* FROM MEAT AND POULTRY PRODUCTS

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9.1 Introduction

Yersinia enterocolitica and other *Yersinia* species such as *Y. frederiksenii* and *Y. kristensenii* are ubiquitous in the natural environment, and may be recovered from water, soil, animals, and food. There is considerable variation within the species *Y. enterocolitica*, and member organisms range from the so-called "*Y. enterocolitica*-like" organisms and "environmental" strains of *Y. enterocolitica* to strains capable of causing serious disease in humans. Hogs have been shown to be a reservoir for certain types of pathogenic *Y. enterocolitica* and pork products have been implicated in human disease. The presence of pathogenic *Y. enterocolitica* on food products is a special concern since those organisms are capable of growth at refrigerator temperatures.

Pathogenic *Y. enterocolitica* organisms are significant causes of human disease in many parts of the developed world. Epidemiological evidence from Belgium, Norway, Denmark, The Netherlands, Japan, Canada, and elsewhere strongly implicates consumption of pork products in human disease. In fact, disease due to *Y. enterocolitica* in the United States may be on the rise, and more information on contamination of meat (especially pork) and poultry is needed.

The term "pathogenic serotype", when used in reference to *Y. enterocolitica*, typically refers to one of 11 O-antigen groups in the *Y. enterocolitica* serotyping scheme. Some strains belonging to these serotypes have been implicated in human disease and have demonstrated pathogenicity in animal models or tissue culture cell invasiveness tests. Until recently, serotypes O:4,32; O:8; O:13a,13b; O:18; O:20; and O:21 have accounted for the majority of pathogenic serotypes recovered in the U.S. Only recently have serotype O:3 organisms been identified as a common cause of yersiniosis in the United States of America. In a recent American survey of hospitalized gastroenteritis patients, 92% of the *Y. enterocolitica* isolates were serotype O:3 while 5% were serotype O:5,27. Serotypes O:3, O:9, and O:5,27 are well-established human pathogens in other areas of the world. The so-called "North American serotypes" of *Y. enterocolitica* (serotypes

O:8, O:13, and O:21) represent a genetically distinct lineage from that of the other pathogenic serotypes.

While the term "pathogenic serotype" is in common usage, several authors have stated that terms such as "pathogenic phenotype", "pathogenic bio-serotype", and "pathogenic bio-serogroup" are more descriptive since they differentiate between pathogenic and nonpathogenic members of a generally pathogenic serotype. Biogrouping, the phenotypic characterization of *Y. enterocolitica*, can serve as a useful indication of the likely pathogenicity of a given strain. Testing for markers of pathogenicity like calcium dependence, crystal violet dye binding, auto-agglutination, and pyrazinamidase activity provide additional information. Markers are not perfectly correlated with pathogenicity but provide useful information under conditions where animal testing is undesirable or impractical.

Virulence in *Y. enterocolitica* is mediated by both chromosomal and plasmid-borne genes. While chromosomal determinants are stable, plasmids containing virulence genes may be lost during culture and confirmational procedures. Temperatures above 30°C are known to cause the loss of virulence plasmids in pathogenic *Y. enterocolitica*, but plasmid loss may also occur under other, less well-defined, circumstances.

Numerous enrichment schemes have been described for the recovery of *Yersinia enterocolitica* from meat samples. These enrichment procedures include cold enrichment for up to a month, direct selective enrichment, or two-step pre-enrichment/selective enrichment procedures. It appears that some enrichment procedures are better suited for the recovery of pathogenic *Y. enterocolitica* than others, though recovery may be influenced by the type of meat product. Even when using an enrichment and plating scheme reported to give good recovery from a particular meat product, considerable variation in recovery may be observed. Methods reported to provide good recovery of pathogenic *Y. enterocolitica* in one part of the world may not work so well in another geographical area, possibly due to differences in levels of *Y. enterocolitica* and competing flora.

Recovery of pathogenic *Y. enterocolitica* is contingent upon a number of factors including: the level of background flora on the product; the amount of background flora coming through enrichment and plating; the level of pathogenic *Y. enterocolitica* present on the sample; the numbers of non-pathogenic *Y. enterocolitica* and non-pathogenic *Yersinia* spp. present on the product; and loss of

virulence factors during enrichment and plating. Furthermore, a recovery method which gives good recovery of one serotype of pathogenic *Y. enterocolitica* may not be suited to other serotypes. In order to recover any of the important pathogenic serotypes of *Y. enterocolitica* which might be present, multiple enrichment broths and plating media are usually recommended for the recovery of the organism from naturally-contaminated foods.

As there is no "universal" enrichment scheme capable of reliably isolating all important pathogenic serotypes of *Y. enterocolitica*, recovering serotypes O:3, O:8, and O:5,27 necessitates the use of parallel procedures. This protocol specifies the use of three separate enrichment procedures in combination with two selective/differential agars. Even with the use of multiple cultural enrichment schemes, however, shortcomings of conventional cultural procedures for the recovery of pathogenic *Y. enterocolitica* undoubtedly result in an under-estimation of the prevalence of this organism in foods and in clinical specimens. A study reported that while 18% of raw pork products were found to contain *Y. enterocolitica* serotype O:3 by two cultural procedures, use of a genetic probe on plated enrichments gave a detection rate of 60%. One of the main difficulties encountered during conventional cultural isolation of pathogenic *Y. enterocolitica* appeared to be overgrowth of small numbers of pathogenic *Y. enterocolitica* by nonpathogenic yersiniae and other microorganisms. The use of conventional cultural procedures for the detection and recovery of pathogenic *Y. enterocolitica* by FSIS sets the stage for a move towards use of genetically-based detection methods.

A great deal of effort must be expended in the recovery and characterization of presumptively-pathogenic *Y. enterocolitica*. Sequential levels of characterization tests include: identification of presumptive *Yersinia*, speciation to *Y. enterocolitica*, biogrouping the *Y. enterocolitica*, followed by testing for pathogenicity markers. *Y. enterocolitica* is more active biochemically at 25°C than at 35-37°C, meaning that disparate results for a given test may be obtained depending on incubation temperature. This characteristic, coupled with the known temperature-sensitivity of the *Y. enterocolitica* virulence plasmid, makes strict adherence to temperature and time requirements a necessity. A word to the reader: although the extensive characterization protocol appears intimidating, the vast majority of non-*Y. enterocolitica* are effectively eliminated with minimal work by the first tier of testing.

The enrichment and characterization procedures described in this protocol are well-documented in the literature. The inclusion of these procedures in the latest edition of the "Compendium of Methods for the Microbiological examination of Foods" is further evidence of their acceptance by the scientific community.

9.2 Equipment, Reagents and Media

9.21 Equipment

a. Sterile scissors, forceps, knives, pipettes, hockey sticks, and other supplies
b. Balance (sensitivity of ± 0.1 g)
c. Inoculating needles and loops
d. Vortex mixer
e. Stomacher™ and sterile stomacher bags
f. Freezer (-70°C)
g. Stereomicroscope and oblique lighting (optional)
h. Incubators capable of holding temperatures at 4 ± 1°C, 25 ± 1°C, 28 ± 1°C, 30 ± 1°C, 32 ± 1°C, 35 ± 1°C and 37 ± 1°C.

9.22 Reagents

a. 0.25% KOH in 0.5% NaCl aqueous solution
b. Crystal violet (85 µg/ml aqueous solution)
c. Sterile mineral oil
d. 1% Ferrous ammonium sulfate (prepare fresh on day of use)
e. Kovacs' reagent
f. Voges-Proskauer (VP) test reagents
g. Oxidase reagent or reagent-impregnated disc/strip
h. Glycerol (sterile)
i. 1 N HCl solution

9.23 Media

a. Irgasan-Ticarcillin-Cholate (ITC) broth
b. Trypticase Soy Broth (TSB)
c. Bile-Oxalate-Sorbose (BOS) broth
d. 0.01 M Phosphate Buffered Saline (PBS, pH 7.6)
e. Cefsulodin-irgasan-novobiocin (CIN) agar (MUST BE MADE ACCORDING TO FORMULATION IN APPENDIX)
f. Salmonella Shigella Deoxycholate Calcium (SSDC) agar
g. Kligler's Iron agar (KIA) slants

h. Simmon's Citrate agar slants
i. Christensen's urea agar slants
j. Lysine decarboxylase medium (0.5% lysine)
k. Ornithine decarboxylase medium (0.5% ornithine)
l. CR-MOX (Congo Red Magnesium Oxalate) agar
m. Methyl Red-Voges Proskauer (MR-VP) broth
n. β-D-Glucosidase test medium
o. Purple broth with 1% filter-sterilized salicin
p. Purple broth with 1% filter-sterilized xylose
q. Purple broth with 1% filter-sterilized sucrose
r. Purple broth with 1% filter-sterilized trehalose
s. Purple broth with 1% filter-sterilized rhamnose
t. Esculin agar slants
u. Sterile Saline (0.85% NaCl)
v. Tween 80 agar (lipase test agar)
w. DNase test agar
x. Tryptophan broth (indole test medium)
y. Pyrazinamide agar slants
z. Veal infusion broth
aa. Trypticase Soy agar or Brain Heart Infusion agar plates

NOTE: Formulations for all the very specialized media and reagents used for the isolation and identification of *Yersinia* are presented at the end of this chapter.

9.3 Isolation Procedures

9.31 Preparation of Sample Homogenate

a. For meat samples other than surface samples: Add 25 g of sample to 100 ml of 0.01 M Phosphate Buffered Saline (PBS: pH 7.6). Homogenize for 2 minutes in a Stomacher™. Allow homogenate to stand undisturbed at room temperature for 10 minutes to allow settling of large meat particles.

b. For carcass surface samples: Add PBS to surface sample so as to prepare a 2:1 ratio of volume to surface area (e.g. add 100 ml PBS to a 50 cm^2 sample). Homogenize for 2 minutes in a Stomacher™. Allow homogenate to stand undisturbed at room temperature for 10 minutes.

9.32 Enrichment & Plating Procedures

In order to improve the chances of recovering pathogenic *Y. enterocolitica*, three enrichment procedures (ITC, TSB/BOS, and PBS) should be used. Although this will increase a laboratory's work-load, it is the best way to insure that any serotype of pathogenic *Y. enterocolitica* present in the product will be recovered. ITC broth provides good recovery of serotype O:3 and probably serotype O:9 *Y. enterocolitica*. TSB/BOS permits recovery of serotype O:8. PBS-cold enrichment has been shown to recover serotype O:5,27. KOH treatment of *Y. enterocolitica* enrichment cultures decreases background flora. Two selective plating media, SSDC and CIN agars, are recommended for the isolation of pathogenic *Y. enterocolitica*. Figure 1 illustrates the enrichment procedures which are included in this protocol.

a. ITC broth: Transfer 2 ml of sample homogenate supernatant into 100 ml ITC broth contained in an Erlenmeyer flask. Incubate at 25°C for 2 days. Spread-plate 0.1 ml onto SSDC agar and incubate the plates at 30°C for 24 h. Spread-plate 0.1 ml onto CIN agar, and incubate the plates at 32°C for 18 h. Also, remove 0.5 ml of the ITC enrichment, treat it with KOH, then streak onto CIN. Reincubate the ITC enrichment at 25°C for another 24 h. After the plate incubation is complete, examine the plates as described below. If colonies having typical *Y. enterocolitica* morphology are not visible on the plates, the ITC culture should be plated out as before.

b. TSB/BOS: Transfer 20 ml of sample homogenate supernatant into 80 ml TSB. Incubate at 25°C for 24 h. Transfer 0.1 ml of the TSB culture into 10 ml BOS. Incubate at 25°C for 3 days. Spread-plate 0.1 ml onto SSDC agar and incubate the plates at 30°C for 24 h. Spread-plate 0.1 ml onto CIN agar, and incubate the plates at 32°C for 18 h. Also, remove 0.5 ml of the BOS enrichment, treat it with KOH, then streak onto CIN. Reincubate the BOS enrichment culture at 25°C for 2 additional days, then plate as before.

c. PBS: Refrigerate the remainder of the PBS homogenate at 4°C for 14 days. Spread-plate 0.1 ml onto CIN agar, and incubate the plates at 32°C for 18 h. Also, remove 0.5

ml of the PBS enrichment, treat it with KOH, then streak onto CIN. Also, use KOH treatment with plating onto CIN.

d. KOH treatment: Add 0.5 ml of enrichment culture to 4.5 ml KOH/NaCl. Vortex briefly (3-4 sec) and IMMEDIATELY streak a loop-full of the KOH-treated broth onto CIN agar (Do NOT use KOH treatment in combination with SSDC agar).

9.33 Selection of Colonies from Plating Media

Due to the fact that SSDC and CIN agars are not completely inhibitory to non-yersiniae, a variety of non-*Yersinia* organisms may be recovered from these agars. Some of these organisms (e.g. strains of *Citrobacter* and *Enterobacter*) have a colonial morphology similar to that of *Y. enterocolitica*. Care must be exercised in the selection of suspect colonies from SSDC and CIN agars in order to minimize picking non-yersiniae. It may be helpful for the analyst to compare colonies growing on sample plates to colonies on the positive control plates. Colony appearance can change over time so strict adherence to time/temperature recommendations is necessary.

a. SSDC: On SSDC, *Y. enterocolitica* colonies are typically round, about 1 mm in diameter and opaque or colorless. When observing plates through a stereomicroscope with oblique transillumination, look for irregular colony edges with a finely granular colony center (never iridescent). Non-yersiniae present either an entire edge or a coarser pattern or both.

b. CIN: On CIN, typical *Y. enterocolitica* colonies have a red bulls-eye which is usually very dark and sharply delineated. The bulls-eye is surrounded by a transparent zone with varying radii, with the edge of the colony either entire or irregular; colony diameter is ca. 1-2 mm (larger colonies are usually not *Yersinia*). Again, the use of a stereomicroscope and oblique transillumination may facilitate examination of plates.

9.4 Identification and Confirmation Procedures

9.41 Identification of *Yersinia*

Select a colony on CIN or SSDC having morphology typical of *Y. enterocolitica* and emulsify colony in about 1 ml of sterile saline (0.85%). Use this to first inoculate a slant of Simmon's Citrate Agar, then inoculate Kligler's Iron Agar, and a tube of urea agar. Repeat until 5 colonies having morphology typical of *Y. enterocolitica* have been selected from each plate of selective agar. Table 1 presents the testing scheme to which isolates recovered from SSDC and CIN will be submitted.

a. Simmon's Citrate: Only Streak-inoculate the slant of a tube of Simmon's Citrate agar; do NOT stab the butt. Incubate at 28°C for 24 h. Presumptive *Y. enterocolitica* are citrate negative (-) and the citrate slant will remain the original green color (a positive (+) reaction is characterized by the agar turning a vivid blue color).

b. Kligler's Iron Agar: Stab-inoculate the butt and streak the slant. Incubate at 28°C for 18-24 h. Presumptive *Y. enterocolitica* should present an alkaline (red) slant and acid (yellow) butt, without gas or H_2S on KIA.

c. Christensen's urea agar: Streak the slant with a heavy inoculum load; do NOT stab the butt. Incubate at 28°C for 24-72 h. Presumptive *Y. enterocolitica* are (+) for urease and will turn the agar to an intense red-pink color.

9.42 Confirmation and Biogrouping of *Yersinia enterocolitica*

Any organism which is citrate negative (-), urease positive (+), and gives an alkaline slant/acid butt without gas or H_2S on KIA should be submitted to further testing. Inoculum for further testing may be obtained from the KIA slant; the KIA slant should then be refrigerated pending the test results. THE TESTS LISTED BELOW ARE ALL NECESSARY TO CONFIRM AND BIOGROUP POTENTIALLY-PATHOGENIC *Y. enterocolitica*. Do NOT attempt to biogroup any isolate until the results are available from ALL tests! Similarly, do NOT discard any culture until ALL tests have been completed. See Holt et al., 1994, for additional information on speciating *Yersinia*.

a. Oxidase test: Test colony growth from the KIA slant of any presumptive *Y. enterocolitica* isolates using oxidase reagent or commercially-available, reagent-impregnated test strips/discs. *Yersinia* are oxidase negative (-).

b. Lysine and ornithine decarboxylase: Inoculate one tube each of lysine decarboxylase medium and ornithine decarboxylase medium; overlay each inoculated tube with sterile mineral oil (4-5 mm deep layer). Incubate at 28°C for 4 days. *Y. enterocolitica* are LYS negative (-) and ORN positive (+).

c. Rhamnose, sucrose, xylose, and trehalose utilization: Inoculate one tube of each of these carbohydrate broths, and incubate at 25°C for 10 days, reading after 1,2,3,7, and 10 days. *Y. enterocolitica* are rhamnose negative (-) and sucrose positive (+). Xylose and trehalose reactions vary between biogroups.

d. Salicin utilization: Inoculate a tube of salicin broth, and incubate at 35°C, reading after 1,2,3, and 4 days. Salicin reactions vary between biogroups.

e. Esculin hydrolysis: Inoculate a tube of esculin agar. Incubate at 25°C for 10 days, reading after 1,2,3,7 and 10 days. Blackening indicates esculin hydrolysis. Esculin reactions vary between biogroups of *Y. enterocolitica*.

f. Indole test: Inoculate a tube of Tryptophan broth (indole test medium). Incubate (with loosened caps) at 28°C for 48 h. Add 0.5 ml of Kovacs' reagent, mix gently, then allow tubes to stand about 10 minutes. A dark red color developing below the solvent layer is evidence of a positive (+) test while the color will remain unchanged in a negative (-) test. Indole test results vary with biogroup of *Y. enterocolitica*.

g. VP test: Inoculate a tube of MR-VP broth, and incubate at 25°C for 24 h. After incubation, add 0.6 ml α-naphthol to the tube, and shake well. Add 0.2 ml 40% KOH solution with 0.3% creatine and shake. Read results after 15 minutes and 1 hour. Development of a pink to ruby red color is a positive test. Results vary with biogroup.

h. β-D-Glucosidase test: Emulsify culture in saline to McFarland 3 turbidity. Add 0.75 ml of culture suspension to 0.25 ml of β-D-glucosidase test medium. Incubate at 30°C overnight (16-20 h). A distinct yellow color indicates a positive reaction. Results vary with biogroup.

i. Lipase test: Inoculate *Y. enterocolitica* isolate onto a plate of Tween 80 agar (more than one isolate may be tested per plate). Incubate at 28°C, and examine after 2 and 5 days. Lipase activity is evidenced by an opaque halo surrounding the streak, and varies with biogroup.

j. Deoxyribonuclease (DNase) test: Inoculate *Y. enterocolitica* strain onto a plate of DNase test agar by streaking the medium in a band (about 3/4 inch length streak). Four or more strains may be tested per plate. Incubate plates at 28°C for 18-24 h. Following incubation, examine plates as follows. For DNase test agar, flood plate with 1 N HCl. A zone of clearing around a colony indicates a positive test. Observe for clear zones surrounding the streak (no clearing or a uniformly opaque agar indicates a negative reaction). DNase test agars containing toluidine blue or methyl green may also be used; follow manufacturer's instructions for interpreting results.

k. Pyrazinamidase test: Inoculate strains over entire slant of pyrazinamide agar and incubate at 25°C for 48 h. Flood slant surface with 1 ml of freshly prepared 1% (w/v) aqueous solution of Fe^{+2} ammonium sulfate. Read after 15 minutes; a pink to brown color indicates PYR positive (+); (presence of pyrazinoic acid) while no color development is observed with PYR negative (-) strains. Pathogenic strains are PYR negative (-).

9.43 Testing for Pathogenicity Markers

Presumptive pathogenic *Y. enterocolitica* are LYS negative (-), ORN positive (+), sucrose positive (+), salicin negative (-) and esculin negative (-). Once the results from all the biogrouping tests are available, Table 2 should be consulted for information on biogroup designation. *Y. enterocolitica* isolates belonging to Biogroups 1B, 2, 3, 4, or 5 should be subjected to further testing for pathogenicity markers.

a. Auto-agglutination in MR-VP broth: Inoculate 2 tubes of MR-VP broth; incubate one at 25°C for 24 h, and the other at 35°C for 24 h. After incubation, the tube incubated at the lower temperature should exhibit turbidity from cell growth. The tube which had been incubated at 35°C should show agglutination (clumping) of bacteria along the walls and/or bottom of tube and clear supernatant fluid. Test is plasmid-dependent.

b. Congo red binding/crystal violet binding: Grow isolates in TSB at 25°C for 16-18 h, then dilute in saline to obtain about 10^4 cfu/ml and dilute to 10^{-5}. Spread-plate 10 µl of diluted suspension on CR-MOX plates. Incubate plates at 37°C for 24 h. A predominance of tiny red colonies is indicative of a positive response for both congo red binding and calcium dependency (some large colorless colonies [CR-MOX negative] may be present due to loss of the virulence plasmid). Perform crystal violet binding on the same agar by flooding each plate with about 8 ml of crystal violet (85 µg/ml), allowing this to stand for 2 minutes, then decanting off the dye. If desired, plates may be observed with a stereo dissecting microscope at 40X magnification. Examine colonies as soon as possible as color tends to fade with time; positive isolates display small, intensely purple colonies. CR-MOX permits demonstration of calcium dependency, Congo red binding, and crystal violet dye binding. Test is plasmid-dependent.

9.5 Method Quality Control Procedures

Due to the variety of bio-serogroups of *Y. enterocolitica* which can be found on meat and poultry, a cocktail of control cultures (including serotypes O:3 and O:8) should be used as a positive control. In addition, an uninoculated media control should be utilized for each of the different enrichment media.

Inoculate control strains into separate tubes of TSB. Incubate at 25°C for 18-24 h. In order to provide ca. 30-300 cfu/ml, make a 10^{-7} dilution of each culture in sterile saline. Add 1 ml of the 10^{-7} dilution of each culture to a single bottle containing 50 ml PBS. Mix well. From this point forward, treat the PBS/*Y. enterocolitica* positive-control cocktail as a sample, following the instructions given above in Section 9.32. Confirm

at least one isolate (of each morphological type present on each of the agars) recovered from the positive-control sample.

9.6 Storage of Isolates

9.61 Maintenance of *Y. enterocolitica* Control Strains

Because of the possibility of plasmid loss in virulent *Y. enterocolitica*, it is recommended that control strains of *Y. enterocolitica* be immediately subcultured upon receipt (incubating at temperatures below 30°C), then preserved in a frozen state.

Inoculate a tube of veal infusion broth with each control strain. Incubate for 48 h at 25°C. Add sterile glycerol to a final concentration of 10% (e.g. 0.3 ml in 3 ml veal infusion broth), dispense into several sterile vials, and freeze immediately at -70°C. Preparation of a batch of vials for each strain is recommended so that one vial can be held in reserve to serve as a source of inoculum for preparation of a new batch of frozen stocks.

When a fresh culture of a control strain is needed, a small portion of frozen suspension may be removed aseptically and transferred to a tube of TSB. Incubation should be at 25°C for 24 h, followed by streaking onto a non-selective agar such as TSA or BHI agar with incubation at 25°C for 24 h.

Strains may be kept on TSA or BHI slants at 4°C for short periods of time, but it is not recommended that such strains be transferred due to the possibility of plasmid loss.

Periodically, control cultures should be tested for pathogenicity markers as described above. Cultures which have lost the virulence plasmid should be destroyed, and replaced by a fresh subculture from the frozen stock preparation.

9.62 Maintenance of Isolates During Confirmation

Due to the possibility of plasmid loss during extensive subculturing (even at temperatures below 30°C), it is recommended that presumptive *Y. enterocolitica* isolates be frozen following *Y. enterocolitica* confirmation testing.

From the KIA slant of a presumptive *Y. enterocolitica* isolate, inoculate a tube of veal infusion broth.

Incubate for 48 h at 25°C. Add sterile glycerol to a final concentration of 10%, and freeze immediately at -70°C.

9.7 Selected References

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Figure 1. Enrichment schemes used for the recovery of pathogenic *Y. enterocolitica* from meat or poultry samples.

Homogenize Sample in PBS

2 ml into 100 ml ITC broth	20 ml into 80 ml TSB	remainder of homogenate
2 days 25°C	1 day 25°C	14 days 4°C
--Onto SSDC 24 h 30°C	--0.1 ml TSB culture + 10 ml BOS 25°C 3 days	--Onto CIN --KOH Onto CIN
--Onto CIN 18 h 32°C	--Onto SSDC --Onto CIN	
--KOH treatment Onto CIN	-KOH treatment Onto CIN	
After 1 additional day[a] of broth incubation	After 2 additional days of broth incubation	
--Onto SSDC	--Onto SSDC	
--Onto CIN	--Onto CIN	
--KOH treatment	--KOH treatment	

Onto CIN Onto CIN

[a] Plating should only be done if colonies having typical *Y. enterocolitica* morphology are not present on plates inoculated on previous day.

Table 1. Sequence of Confirmation, Biogrouping, and Pathogenicity-marker Tests used for *Y. enterocolitica*

Yersinia Confirmation Tests	Simmons' Citrate slant	Kligler's Iron Agar slant & butt	Christensen's urea agar
	28°C, 24-72 h	28°C, 18-24 h	28°C, 18-72 h
	Citrate (-) (green) little/no gas	Alk/Acid no H_2S	Urea (+) (pink)
Y. enterocolitica Confirmation Tests	Oxidase Lysine decarboxylase Ornithine decarboxylase Rhamnose utilization Sucrose utilization		
Y. enterocolitica Biogrouping Tests	Lipase DNase Indole Xylose VP β-D-Glucosidase		

	Pyrazinamidase	
	Salicin;	Esculin
	Trehalose;	Nitrate Reduction
Pathogenicity- Marker Tests	Autoagglutination in MR-VP broth	
	Congo Red Binding	
	Crystal Violet Binding	

Table 2. Biogrouping Scheme for *Yersinia enterocolitica* [a]

	Biogroups[b]					
	1A	1B[c]	2[c]	3[c]	4[c]	5[c]
Lipase (Tween-esterase)	+	+	-	-	-	-
Esculin/salicin 24 h[d]	+,-	-	-	-	-	-
Indole	+	+	(+)[e]	-	-	-
Xylose	+	+	+	+	-	V[f]
Trehalose/NO_3[g]	+	+	+	+	+	-
Pyrazinamidase	+	-	-	-	-	-
β-D-Glucosidase	+	-	-	-	-	-
Voges-Proskauer	+	+	+	+[h]	+	(+)
DNase	-	-	-	-	+	+

[a] Modified from Wauters et al., 1987.

[b] Reactions from tests incubated at 25-28°C, with the exception of β-D-Glucosidase which was incubated at 30°C and salicin which was incubated at 35°C. Incubation at other temperatures may result in different results and biogroupings.

[c] Biogroup contains pathogenic strains.

[d] Esculin and salicin reactions for a given strain of *Y. enterocolitica* are nearly always identical so they are listed together in this table.

[e] Indicates a delayed positive reaction.

[f] Indicates variable reactions.

[g] Trehalose and nitrate reduction reactions for a given strain of *Y. enterocolitica* are nearly always identical so they are listed together in this table.

[h] Rarely, a serotype O:3 strain may be negative for VP.

ADDENDUM

Formulations for Media and Reagents for *Yersinia enterocolitica* Isolation and Identification

β-D-Glucosidase test

Add 0.1 g 4-nitrophenyl-β-D-glucopyranoside to 100 ml 0.666 M NaH_2PO_4 (pH 6.0), dissolve, then filter-sterilize.

BOS broth

$Na_2HPO_4*7H_2O$	17.25 g
Na oxalate	5.0 g
Bile salts No. 3 (Difco)	2.0 g
NaCl	1.0 g
0.1% solution of $MgSO_4*7H_2O$	10.0 ml
Distilled deionized H_2O	639.0 ml

Combine ingredients and mix until dissolved, adjust pH to 7.6 with 5 N HCl, then autoclave at 121°C for 15 minutes.

Add the following filter-sterilized solutions:

100 ml of 10% sorbose
100 ml of 1.0% asparagine
100 ml of 1.0% methionine
10 ml of 2.5 mg/ml metanil yellow
10 ml of 2.5 mg/ml yeast extract
10 ml of 0.5% Na pyruvate
1 ml of 0.4% solution of Irgasan DP300 (2,4,4'-trichloro-2'-hydroxydi

Adjust pH to 7.6 with either 5 N NaOH or HCl as required. Store at 4°C for up to 7 days.

On day of use, add 10 ml of 1.0 mg/ml Na furadantin (from stock solution stored at -70°C) to the above complete base. Aseptically dispense 10 ml portions into sterile tubes.

CIN agar

MUST CONTAIN Cefsulodin at 4 mg/L:

This formulation is commercially available from Difco; premixes available from other manufacturers contain different levels of cefsulodin.

Oxoid special peptone	20.0 g
Yeast extract	2.0 g
Mannitol	20.0 g
Na pyruvate	2.0 g
NaCl	1.0 g
0.1% aqueous stock solution of $MgSO_4*7H_2O$	10.0 ml
Na deoxycholate	0.5 g
Oxoid No. 4 (L11) agar	12.0 g
Distilled deionized H_2O	748.0 ml

Bring to a boil in order to dissolve agar completely (do NOT autoclave). Cool to around 80-85°C.

Add 10 ml of Irgasan DP300 (2,4,4'-trichloro-2'-hydroxydiphenyl ether, Ciba Geigy) solution (0.04% in 95% ETOH). Shake vigorously to disperse ethanol. Cool in a water bath to ca. 50-55°C.

Add 1 ml of 5 N NaOH, then 10 ml of each of the following aqueous, filter sterilized (0.22 µm pore size) stock solutions:

neutral red (3 mg/ml)
crystal violet (0.1 mg/ml)
cefsulodin (0.4 mg/ml)
novobiocin (0.25 mg/ml).

[Stock antibiotic solutions are stored at -70°C and thawed at room temperature just before use]

Adjust final pH to 7.4 with 5 N NaOH. Store prepared plates at around 20-25°C for up to 9 days.

CR-MOX agar

Tryptic soy agar	40.0 g
Distilled deionized H_2O	825.0 ml

Mix and autoclave at 121°C for 15 minutes. Cool basal medium to 55°C.

Add the following solutions:

a) 80 ml of 0.25 M sodium oxalate (Sigma) solution (sterilized by autoclaving at 121°C for 15 minutes)
b) 80 ml of 0.25 M magnesium chloride solution (sterilized by autoclaving at 121°C for 15 minutes)
c) 10 ml of 20% D-galactose solution (sterilized by autoclaving at 115°C for 10 minutes)
d) 5 ml of 1% Congo red solution (sterilized by autoclaving at 121°C for 15 minutes)

Mix well and dispense into 15 X 100 mm petri dishes. Store prepared media in plastic bags at 4°C for up to 3 months.

DNase test Agar

Tryptose	20.0 g
Deoxyribonucleic acid	2.0 g
Sodium chloride	5.0 g
Agar	15.0 g
Distilled water	1.0 L

Suspend all ingredients and heat to boiling to dissolve completely. Sterilize in the autoclave at 121°C for 15 minutes, final pH = 7.3. Dispense into sterile Petri dishes.

Esculin agar

Polypeptone (Oxoid)	10.0 g
Esculin	1.0 g
Ferric ammonium citrate	1.0 g
Agar	5.0 g
Distilled deionized H_2O	1.0 L

Mix well. Dispense into tubes, and autoclave at 121°C for 15 minutes.

Indole test medium

Prepare a 1% solution of Bacto Peptone (Difco) OR 1% Trypticase peptone (BBL) OR use Tryptone Water (Oxoid). Dispense 5 ml quantities into tubes. Sterilize by autoclaving at 121°C for 15 minutes.

ITC broth

Tryptone	10.0 g
Yeast extract	1.0 g
$MgCl_2*6H_2O$	60.0 g
NaCl	5.0 g
0.2% (w/v) malachite green solution (aqueous)	5.0 ml
$KClO_3$	1.0 g
Distilled deionized H_2O	1.0 L

Mix above ingredients, autoclave at 121°C for 15 minutes, cool. Then add,

a) 1 ml of Ticarcillin solution (1 mg/ml in H_2O; filter-sterilized) (Ticarcillin available from Sigma)
b) 1 ml of Irgasan DP300 (1 mg/ml in 95% ethanol); AKA 2,4,4'-trichloro-2'-hydroxydiphenyl ether (CIBA-Geigy, Basel)
c) Mix well. Dispense 100 ml into sterile 100 ml Erlenmeyer flasks (it is important to minimize the surface area:volume ratio). Store at 4°C for up to 1 month.

Kligler's iron agar (KIA) slants

Polypeptone peptone	20.0 g
Lactose	20.0 g
Dextrose	1.0 g
NaCl	5.0 g
Ferric ammonium citrate	0.5 g
Sodium thiosulfate	0.5 g
Agar	15.0 g
Phenol red	0.025 g
Distilled water	1.0 L

Heat with agitation to dissolve completely. Dispense into 13 X 100 mm screw-cap tubes and autoclave for 15 minutes at 121°C. Cool and slant to form deep butts. Final pH = 7.4.

KOH solution

NaCl	5.0 g
KOH	2.5 g
Distilled deionized H_2O	1.0 L

Dispense 4.5 ml amounts in small screw-cap tubes, and sterilize at 121°C for 15 minutes. Tighten caps when cool. Make only a small number of tubes at a time since pH decreases with storage time; store at 4°C for no more than 7 days.

Pyrazinamide agar

Tryptic soy agar (Difco)	30.0 g
Pyrazine-carboxamide (Merck)	1.0 g
0.2 M Tris-maleate buffer (pH 6)	1.0 L

Mix well, dispense 5 ml amounts in tubes (160 X 16 mm). Autoclave at 121°C for 15 minutes. Slant for cooling.

SSDC agar

SS agar (quantity per liter as stated by a particular Manufacturer)

Yeast extract	5.0 g
Na deoxycholate	10.0 g
$CaCl_2$	1.0 g
Distilled deionized H_20	1.0 L

Adjust pH to 7.2 to 7.3 Bring agar almost to a boil on a hot plate (Do NOT autoclave). Temper agar to 55-60°C, mix and pour while still warm, making thick plates. Store prepared plates for 7 days at 20-25°C in the dark. Do NOT store at 4°C.

Tween 80 agar (Lipase test agar)

Peptone	10.0 g
NaCl	5.0 g
$CaCl_2$*H_2O	0.1 g
Agar	15.0 g
Distilled deionized H_2O	1.0 L

Sterilize agar base by autoclaving at 121°C for 15 minutes. Temper to 45-50°C.

Sterilize Tween 80 by autoclaving at 121°C for 20 minutes.

Add sterile Tween 80 to tempered agar base to give a final concentration of 1% (v/v). Mix well. Dispense into Petri dishes, and allow to solidify.

Veal infusion broth

Veal, infusion from	500.0 g
Proteose peptone # 3	10.0 g
NaCl	5.0 g
Distilled water	1.0 L

Heat with agitation to dissolve all ingredients. Dispense 7 ml portions into 16 X 150 mm tubes and autoclave at 121°C for 15 minutes. Final pH = 7.4.